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13. ABSTRACT *(Maximum 200 Words)*

Dysregulation of the control of both cellular proliferation and cell death contributes to tumor growth. Consistent with this hypothesis, overexpression of genes that block apoptosis is observed in tumors. Furthermore, upregulation of genes that confer survival results in an increased resistance of tumors to apoptosis and thereby renders them resistant to many chemotherapeutic drugs. The inhibitor of apoptosis proteins (IAPs) are a family of anti-apoptotic proteins that are conserved across species. This family of proteins includes six human members, including c-IAP1/HIAP2, c-IAP2/HIAP1, XIAP/HILP, survivin, NAIP, and BRUCE. We have shown that the IAPs are direct inhibitors of specific members of the cysteine family of cell death proteases, caspases-3, -7, and -9. The caspases play a central role as effectors in the apoptotic cascade and their inhibition contributes to tumor growth and resistance to chemotherapeutic agents. We have found that IAPs are over-produced in some types of cancer, including adenocarcinomas and the breast. These observations lay the foundation for eventually abrogating the effects of IAPs and restoring apoptosis sensitivity to cancers.

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Manyla Krajewska 3.17.2000

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Annual Report
DAMD17-97-1-7294

Maryla Krajewska, MD

The Role of IAP Family Genes in Breast Cancer

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INTRODUCTION

Apoptosis is a regulated cell suicide program that plays a critical role during developmental and adult tissue homeostasis. Excessive apoptosis results in severe cell loss, such as that observed in degenerative disorders, while insufficient apoptosis contributes to the pathogenesis of disorders such as cancer (1). The Inhibitor of Apoptosis Proteins (IAPs) constitutes a family of anti-apoptotic proteins that are conserved across species. The first human IAP to be identified, the Neuronal Apoptosis Inhibitor Protein (NAIP), was isolated based on its contribution to the neuromuscular disorder, spinal muscular atrophy (SMA) (2). This family of proteins now includes six human (c-IAP/HIAP2; c-IAP/HIAP1; XIAP/hILP; Survivin, NAIP; BRUCE), two *Drosophilia* (DIAP1; DIAP2), two yeast (SpBIR1, ScBIR2) and two *c. elegans* homologs. The common structural feature of all family members is a motif termed the baculovirus IAP repeat (BIR) that is present in two to three copies. Theses motifs, at least for the mammalian, viral and *Drosophilia* homologs, are sufficient to mediate cell survival. The human c-IAP1 and c-IAP2 proteins are unique in that they are recruited to the cytosolic domains of Tumor Necrosis Factor Receptors via their association with the TNF-associated factor (TRAF) proteins, TRAF-1 and TRAF-2. However, the role that the IAPs play in these signal transduction pathways is not fully understood.

The cysteine family of proteases, named caspases, play a central role as effectors in the apoptotic cascade with apoptotic stimuli leading to proteolytic processing of upstream caspases that activate distal caspases either directly or indirectly through a mitochondrial step. These active caspases cleave key regulatory and structural proteins, thus leading to the demise of the cell. Disruption of mitochondrial homeostasis is central to the induction of cell death. Cytochrome c redistribution into the cytosol, calcium flux, and loss of transmembrane potential are the biochemical perturbations that lead to downstream caspase activation and death. Cytochrome c release has been shown to precede the loss of mitochondrial transmembrane potential and caspase activation.

It is well established that tumor cells have a decreased ability to undergo apoptosis and often this occurs due to an upregulation of genes that confer a survival advantage. Chemotherapeutic drugs normally induce regression by activation of caspases, thus an upregulation of genes that inhibit these proteins renders tumors resistant to these treatments. The viral IAPs were isolated based on their ability to functionally complement the viral caspase-inhibitory protein p35 (3). We hypothesized that the IAPs could promote cell survival by a similar mechanism and thus proposed experiments to test this idea and to understand the relevance of IAPs to breast cancer.

BODY

OBJECTIVES:

The originally proposed objectives remain applicable and are being systematically accomplished.

1. Analyze IAP expression in normal breast tissue, and breast tumor specimens and cell lines.
2. Determine the functions of IAPs in breast cancer cell lines.
3. Determine what other proteins IAPs bind.
4. Examine the effects of IAPs on caspase-family cell death proteases.

PROGRESS:

We have continued to make outstanding progress towards the originally proposed objectives. Objectives #2 and #4 were completed and have been described in previous progress reports. Objective #1 was the major focus of the past funding period. Assays for accomplishing Aim #3 were established and the work is ongoing.

TECHNICAL OBJECTIVE #1

Analyze IAP expression in normal breast tissue, and breast tumor specimens and cell lines.

Task #1: Raise antibodies to IAPs.

Accomplished and described in an earlier progress report.

Task #2: Northern blot and Western blot analysis of IAP expression in cancer cell lines.

This task was completed during the past funding period resulting in a paper which has been accepted for publication in *Clinical Cancer Research*. A complete copy of the paper with figures is provided as an appendix item. Only a brief summary, therefore, will be provided here.

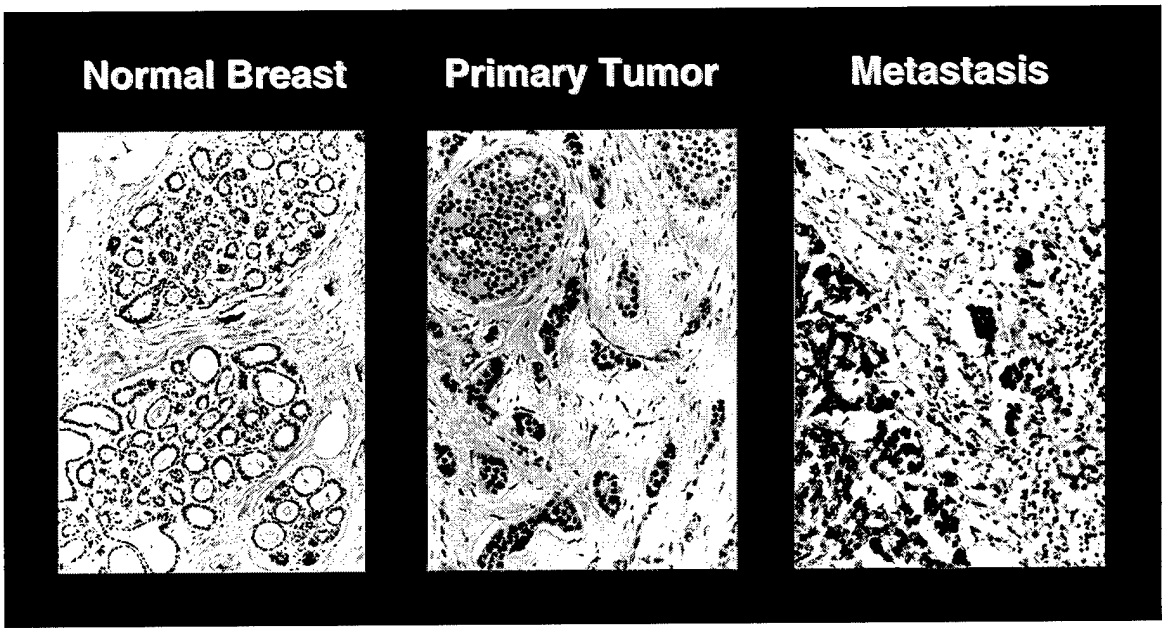
Briefly, a RNase protection assay was established for assessing the relative levels of IAP-family mRNAs in tumor cell lines. Using this assay, we analyzed the NCI 60 tumor cell line screening panel. This analysis showed that cIAP1 and XIAP are widely expressed in human cancer cell lines, while NAIP and cIAP2 are only rarely seen. Correlations of these IAP mRNA levels with other biomarkers previously assessed in the NCI screening panel failed to reveal any correlations (not shown).

Using our antisera specific for cIAP1, cIAP2, XIAP, and Survivin, we phenotyped the NCI 60 tumor cell line screening panel, using a semi-quantitative immunoblotting method (4, 5). The NCI tumor panel was supplemented in some instances with additional cell lines of our own, which increased the number of breast and prostate cancers in the analysis (6). cIAP1 protein levels correlated with tumor resistance to anticancer drugs, suggesting this anti-apoptotic protein is a determinant of chemoresistance.

Task #3: Immunohistochemistry of paraffin embedded breast carcinomas.

We have developed antibodies and procedures for immunohistochemical analysis of cIAP1, cIAP2, XIAP, and Survivin using our antisera and archival paraffin-embedded tumor specimens.

Figure 1 shows examples of Survivin immunostaining in normal mammary epithelium (*left*) and primary tumor (*middle*), and metastatic breast cancer (*right*). Note that normal breast does not express Survivin. In contrast, intense Survivin immunostaining is present within the cytosol of essentially all the invasive breast cancer cells within the primary tumor specimen and in the metastatic breast cancer cells. Similar results have been obtained for 34 primary breast cancers and 6 metastatic lesions. We conclude, therefore, that tumor-specific deregulation of Survivin expression occurs commonly in breast cancers, suggesting an important role for this IAP-family member in this disease.



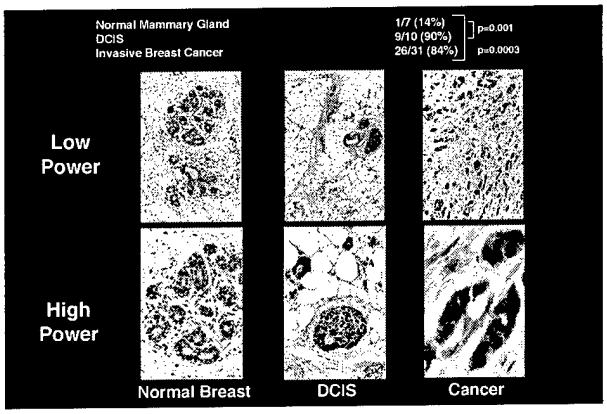


Figure 2 shows results from immunostaining of breast cancer specimens using our XIAP antibody. In contrast to Survivin which is entirely absent from normal breast, XIAP is expressed at low levels in mammary gland. However, comparisons of the intensity of immunostaining of normal mammary epithelial cells with ductal carcinomas in situ (DCIS) and invasive cancer revealed that XIAP levels are apparently upregulated in many breast neoplasms.

Task #4: Analyze bank of tumors previously typed for expression of p53, ER, Bcl-2, Bax, and erbB2 for levels of IAP expression.

In Progress.

TECHNICAL OBJECTIVE #2. Functional analysis of IAPs in breast cancer cell lines.

Task #5: Generation of IAP stable cell lines.

Accomplished and described in an earlier progress report.

Task #6: Cell death assays with stable cell lines to assess effects of IAPs.

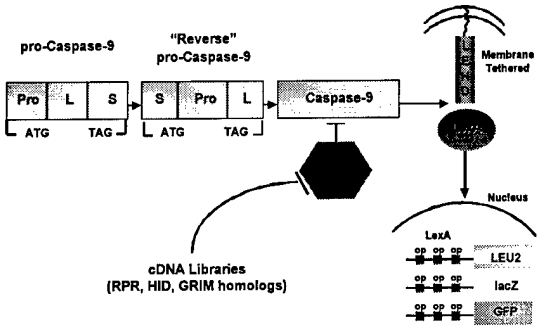
Accomplished and described in an earlier progress report.

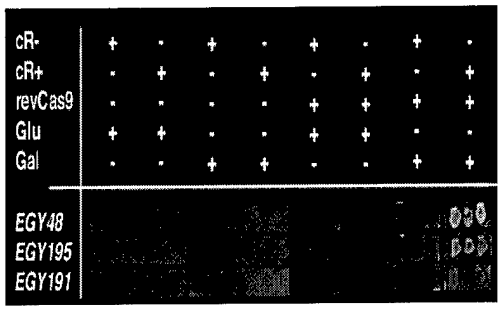
TECHNICAL OBJECTIVE #3. Protein/Protein Interaction Studies.

Task #7: Yeast two hybrid assay to isolate interacting proteins.

Two cDNA libraries were screened with cIAP1 and cIAP2 (full-length) or the BIR-domain regions of these proteins as baits. Several positive clones were obtained, all representing components of the ubiquitin/proteosome machinery. Attempts were made to examine whether IAPs somehow regulate the proteosome, using purified preparations of 26S proteosome and measuring proteosome-mediated degradation of a substrate peptide. No consistent effect of IAPs were observed. Our current hypothesis is that the levels may be regulated in yeast by ubiquitination and proteosome-mediated degradation, thus explaining why they interact with ubiquitin/proteosome cDNAs in two-hybrid assays. We are exploring whether IAPs are similarly regulated by ubiquitination/degradation in human cells.

Because of the lack of success with yeast two-hybrid assays, we developed an alternative functional cloning strategy in hopes of identifying novel IAP-interacting proteins. The approach is modeled after the knowledge about how IAPs are regulated in Drosophila (flies), where the death proteins rpr, hid, and grim have been shown to bind IAPs and prevent them from repressing caspases (reviewed in 7). We seek human homologues or equivalents of these IAP-inhibiting proteins, since studies of such proteins are likely to reveal mechanisms for abrogating the effects of IAPs in breast cancers. We therefore, devised a functional cloning method in yeast. Active caspase-9 is expressed in yeast using the technique of circular permutation. We constructed a membrane-tethered version of the transcription factor LexA, containing a cleavable linker. Cleavage of this LexA version protein by caspase-9 releases LexA from the plasmid membrane and allows it to enter the nucleus and transactivate reporter plasmids containing LexA operators, including LEU2, LacZ, and Green Fluorescent Protein (GFP) reporters. The next step will be to repress caspase-9 with IAPs, then to screen breast cancer cDNA libraries for proteins that can bind to and suppress IAPs, thus freeing caspase-9 to cleave the LexA reporter. Figure 3 presents the concept for this screen. Figure 4 shows results in which a cleavable LexA reporter (but not a noncleavable reporter) is activated by active ("reverse") caspase-9, thus activating a GFP reporter plasmid in three of three transformants tested.





CR- = Non-Cleavable Reporter
CR+ = Cleavable Reporter
Glucose = Represses Caspase-9 Expression
Galactose = Induces Caspase-9 Expression

Task #8: Isolation of associated proteins by co-immunoprecipitations.

These results were previously reported in an earlier progress report and will not be reiterated here (8-11).

Task #9: Isolation of full-length cDNAs from novel proteins.

Not applicable.

Task #10: Confirmation of interactions in vitro and in mammalian cells.

Much of these results were published previously (8-11)

Task #11: Co-transfection assays with IAPs and novel proteins to determine functional consequence of interaction.

Not Applicable. No novel IAP-binding proteins have been discovered. Co-transfection studies were performed where caspases were overexpressed and IAPs were shown to prevent caspase-induced apoptosis in transient transfection assays. Those findings were previously reported in progress reports and thus will not be reiterated here.

Task #12: Generation of stable cell lines expressing IAP-binding proteins.

Not Applicable.

TECHNICAL OBJECTIVE #4. Explore effects of IAPs on caspase family proteases using caspase activity assays.

Task #13: Caspase assay with tetrapeptide substrates.

Accomplished and described in an earlier progress report.

PLANS

Our plans remain essentially unchanged from the original proposal. We envision that most of the originally proposed objectives should be accomplished within the final year of the grant.

PUBLICATIONS ATTRIBUTED TO THIS GRANT PERIOD

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Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. *Clin. Cancer Res.* In Press. 2000

CONCLUSION

Insufficient apoptosis may result in tumor progression and consequently, the dysregulation of genes that normally inhibit the apoptotic cascade contributes to carcinogenesis. Moreover, tumor resistance to chemotherapeutic drugs has been associated with the upregulation of anti-apoptotic genes. Chemotherapeutic drugs kill by initiating apoptosis and specifically lead to increases in caspase activity. We have shown that IAPs inhibit caspases. Pharmacological agents that induce cell death by the specific targeting of these should contribute to tumor regression by liberating caspases to induce apoptosis.

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Expression and Prognostic Significance of IAP-Family Genes in Human Cancers and Myeloid Leukemias

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Abstract

Expression of several Inhibitor of Apoptosis Proteins (IAPs) was investigated in the NCI panel of 60 human tumor cell lines and the expression and prognostic significance of one of these, XIAP, was evalulated in 78 previously untreated patients with acute myelogenous leukemia (AML). XIAP and c-IAP1 were expressed in most cancer lines analyzed, with substantial variability in their relative levels. In contrast, NAIP mRNA was not detectable, and c-IAP2 was found at the mRNA and protein levels in only 34 (56%) and 5 (8%) of the 60 tumor cell lines analyzed, respectively. Interestingly, XIAP, cIAP1 and cIAP2 mRNA levels did not correlate with protein levels in the tumor lines, indicating post-transcriptional regulation of expression. High levels of XIAP protein in tumor cell lines were unexpectedly correlated with sensitivity to some anticancer drugs, particularly cytarabine and other nucleosides, whereas higher levels of cIAP1 protein levels were associated with resistance to several anti-cancer drugs. The relevance of XIAP to *in vivo* responses to cytarabine was explored in AML, making correlations with patient outcome (n = 78). Patients with lower levels of XIAP protein had significantly longer survival (median 133 vs. 52.5 weeks, P=0.05) and a tendency toward longer remission duration (median 87 vs. 52.5 weeks, P=0.13) than those with higher levels of XIAP. Altogether, these findings show that IAPs are widely but differentially expressed in human cancers and leukemias, and suggest that XIAP protein levels may have adverse prognostic significance for patients with AML.

Introduction

Suppression of apoptosis contributes to carcinogenesis by several mechanisms, including prolonging cell lifespan thus facilitating the accumulation of gene mutations, permitting growth factor-independent cell survival, promoting resistance to immune-based cytotoxicity, and allowing disobeyance of cell cycle checkpoints which would normally induce apoptosis (1-4). Defects in apoptotic mechanisms also play an important role in resistance to chemotherapy and radiation (1).

The IAPs are a family of anti-apoptotic proteins which are conserved across evolution, with homologues found in both vertebrate and invertebrate animal species (5). The baculovirus IAPs, Cp-IAP and Op-IAP, were the first members of this family to be identified based on their ability to functionally complement defects in the cell death inhibitor, p35, a baculovirus protein that binds to and inhibits caspase-family cell death proteases (10,11). Subsequently, five human (XIAP, c-IAP1, c-IAP2, NAIP and Survivin) and two *Drosophila* IAP homologs have been identified, which have been demonstrated to inhibit cell death (4, 12-18). The human IAPs, XIAP, c-IAP1 and c-IAP2 have been reported to bind and potently inhibit caspases–3 and –7 with Ki's in the range of 0.2-10 nM (8, 24). These IAP-inhibitable caspases operate in the distal portions of apoptotic protease cascades, functioning typically as effectors rather than initiators of apoptosis (20, 22, 23). Though quantitative studies are lacking, the IAP-family member Survivin also binds and inhibits some effector caspases (18, 30). Moreover, at least some IAPs, such as XIAP, are capable of binding and suppressing specific initiator caspases such as caspase-9, the pinnacle caspase in the cytochrome-c/mitochondrial pathway for apoptosis (31).

The common structural feature of all IAP family members is an approximately 70 amino acid zinc-binding fold termed the Baculovirus IAP Repeat (BIR) domain, which is present in one to three copies (32). Using a mutagenesis approach, we previously showed that the second of the three BIR domains (BIR2) of XIAP is necessary and sufficient for inhibiting the effector caspases-3 and -7 (19), implying that a single BIR domain can possess anti-apoptotic activity. However, for caspase-9 suppression by XIAP, the third BIR domain is required in combination with an adjacent region comprising a RING zinc-finger domain (38).

To learn more about the importance of IAPs in cancer and leukemia, we examined the expression of several members of the IAP family in the well-characterized NCI panel of 60 human tumor cell lines, correlating their expression at either the mRNA or protein levels with other tumor-related genes and with *in vitro* chemosensitivity data for 30,000 compounds. In addition, the prognostic significance of one of the IAPs, XIAP, was examined in acute myelogenous leukemia, revealing correlations with clinical outcome, thus suggesting that analysis of IAP-family proteins may provide predictive information about responses to chemotherapy and survival for at least some subgroups of patients with AML.

Materials & Methods

Patient Materials. Patient-derived AML-samples (n=97) were randomly selected from a large assortment of patient samples collected, ficoll-purified, and cryopreserved between 1983 and 1995. Only samples with over 15% blasts were selected for further analysis (n=78), to avoid contributions from contaminating normal cells. The median follow up of survivors is nearly five years. Clinical characteristics of these patients are presented below.

Immunoblot Analysis of AML-Samples. Immunoblotting of AML patient samples derived from 78 newly diagnosed patients with AML and 10 normal individuals was carried out using cell lysates from mononuclear fractions of peripheral blood generated by ficoll separation. Whole cell lysates from 5×10^5 cells were electrophoresed through 8%-14% SDS-PAGE gradient gels and electroblotted to Immobilon PVDF membranes (Millipore, Bedford, Mass) using a semi-dry transfer apparatus at 0.8 mA/cm^2 for 1.5 hours (25). Each gel included a XIAP-expressing positive control cell line (HeLa), 2-3 peripheral blood mononuclear cells (PBMC) samples from normal individuals, and molecular weight markers. The membrane was blocked in Tris-buffered saline with 0.05% Tween-20 (TBST) and 3% nonfat dry milk (blotto) at 4°C for 4 hours, then exposed overnight to a mouse anti-XIAP monoclonal antibody (Pharmingen, San Diego, CA) at a 1:350 dilution at 4°C overnight. Subsequently, the membranes were washed twice in blotto, exposed to sheep anti-mouse IgG conjugated to horseradish peroxidase (1:4000) for 1 hour, washed in blotto and TBST, then exposed to SuperSignal® West Pico substrate

chemiluminescence mixture for 1 minute according to the directions of the manufacturer (Pierce, Rockford, ILL). Images were collected on a ChemiImager 4400 system (Alpha Innotech, San Leandro, CA) and densitometry was performed using the best image. To normalize for variation in antibody concentration or time of exposure, the XIAP signal from the patient was normalized against the XIAP signal of the control cell line HeLa. Results are expressed in terms of this ratio.

Immunoblot Analysis of Tumor Cell Lines. Detergent-lysates were prepared in the presence of protease inhibitors from established tumor cell lines essentially as described (6). After normalization for total protein content (50 μg per lane), samples were subjected to SDS-PAGE/immunoblot analysis using monoclonal anti-IAP antibodies specific for XIAP (Transduction Laboratories, Lexington, KY) or for cIAP1 and cIAP2 (R&D Systems, Minneapolis, MN). Data on X-ray films were quantified by scanning-densitometry using the IS-1000 image analysis system (Alpha Innotech Co.) and the results from a standard-curve generated using purified recombinant GST-XIAP, GST-cIAP1, or GST-cIAP2 protein were used to estimate the amounts (ng) of XIAP, cIAP1 and cIAP2 protein per 50 μg total protein. Data from two independent protein standard-containing blots were within 10% agreement.

RNase Protection Assays. Total RNA was isolated from the NCI 60 tumor cell line panel or freshly isolated AML cells using the RNeasy Mini Kit (Quiagen, Germany), according to the manufacturer's manual. After normalization for total mRNA content (12 μg per lane), samples were subjected to RNase Protection Assay, as described in the

manufacturer's manual (Riboquant, Pharmingen, La Jolla, CA). Briefly, a multiprobe was used for the T7 polymerase-directed synthesis of a ^{32}P-labeled anti-sense RNA probe set. The probe set was hybridized in excess to target RNA in solution, after which free-probe and other single-stranded RNA were digested with RNases. The remaining "RNase-protected" probes were purified, resolved on denaturing polyacrylamide gels (4.75%) and quantified by autoradiography and phosphor-imaging (Bio-Rad Laboratories, Inc., Molecular Analyst, Version 2.1). The quantity of each mRNA species in the original RNA sample was then determined based on the intensity of the appropriately sized, protected probe fragment relative to the loading control (GAPDH).

Statistical Analysis. Comparison of IAP mRNA and protein levels with chemosensitivity data for the 60 cell line panel (NCI) resulted in a rank order of drugs from the standard agent database of the NCI. Pearson correlation coefficients and two-tail p-values were used to assign possible significance to these data. A p-value of ≤ 0.005 was considered significant in all analyses.

For analysis of patient data, results were rank-ordered according to XIAP protein levels and divided in to thirds, thus assessing possible threshold effects of XIAP on survival, while avoiding searching for optimal cut-points for levels of XIAP (28). The top two-thirds demonstrated similar outcomes, and therefore those arms were collapsed. Unadjusted survival analyses were performed using Kaplan-Meier plots (29) and comparisons of survival between patient subgroups were made using the log-rank test (26). For comparisons of clinical variables between patients within the lowest third of XIAP

expression versus the top two-thirds, either Student's t-test or χ^2 analysis were performed. All computations were performed using Statistica® version 5.1 M (StatSoft Inc, Tulsa OK.).

Results

IAP mRNA Levels in the NCI Panel of Tumor Cell Lines. We analyzed the levels of XIAP, NAIP, c-IAP1 and c-IAP2 mRNAs in the NCI 60 tumor cell line panel by RNase Protection Assay (RPA). Examples are shown in Figure 1. GAPDH was included as a control to ensure equal loading. Data on X-ray films were quantified by scanning-densitometry (Figure 2A-C). The mRNAs for XIAP and c-IAP1 were detectable at variable levels in all cell lines analyzed (Figure 2A, 2B). In contrast, c-IAP2 was more restricted in its expression. c-IAP2 mRNA was abundant, for example, in most CNS and many renal tumor cell lines, but almost undetectable in ovarian and breast cancer lines (Figure 2C). Thus, in contrast to XIAP and cIAP1 mRNAs which were present in all tumor lines, the presence of cIAP2 mRNA was detectable in only 34 (56%) of the 60 tumor cell lines within the NCI screening panel. NAIP mRNA was undetectable in the entire panel of 60 tumor cell lines, but was plentiful in some normal tissues such as peripheral blood lymphocytes (not shown).

IAP Protein Levels in the NCI Panel of Tumor Cell Lines. To compare the relative levels of IAP mRNAs and proteins in the 60 cell line panel, we analyzed the relative levels of XIAP, cIAP1 and cIAP2 proteins by immunoblot assay using monoclonal antibodies. Data on x-ray films were quantified by scanning-densitometry and compared with results obtained from defined amounts of recombinant protein (standard-curve) to estimate the amount (ng) of IAP protein per 50 μg total protein. Examples of immunoblot data are shown in Figure 3A. Higher levels of XIAP protein were generally found in renal cancer and

melanoma cell lines, whereas lower levels of XIAP were typically present in CNS tumor cell lines (Figure 3B). cIAP1 protein was expressed at higher levels in colon cancers, whereas lower levels were found in melanoma cell lines (Figure 3B). The expression of c-IAP2 was far more restricted; c-IAP2 protein was detectable in only 5 (8%) of the 60 tumor cell lines analyzed (MCF7, HT29, A549, NCI H322M, K562). Thus, protein data for cIAP2 did not correlate with mRNA data.

Similarly, XIAP and cIAP1 protein levels did not correlate with mRNA levels. For example, XIAP mRNA levels were above the mean for the NCI 60 cell screening panel in the leukemia lines HL60 and RPMI-8226, the lung cancer cell lines NCI-H322M and NCI-H460, the colon lines COLO 205 and HCC-2998, the brain tumor lines SF-539 and SNB-75, and the breast cancer lines MCF7 and HS 578T, whereas XIAP protein levels were below the mean in these same tumor lines (compare Figures 2A and 3B). Conversely, XIAP mRNA levels were below the mean of tumor cell lines in the leukemia lines K562 and MOLT4, the lung cancer line HOP-62, the colon cancer line KM12, the melanoma cell lines LOX-IMVI, MALME-3M and M14, the ovarian line OVCAR-3, the renal cancer line 786-0, ACHN, CAKI-1, and TK10, the prostate cancer PC3, and the breast cancer line MDA-MB 231, whereas XIAP protein levels were above the mean within these same tumor cell lines.

Correlation of IAP Protein Levels with Chemosensitivity Data from the NCI 60 Cell Screening Panel. XIAP and cIAP1 protein levels were correlated with cytotoxicity data for compounds tested against the NCI 60 cell screening panel. Since cIAP-2 protein was found in only 5 cell lines, it was excluded from statistical analysis. Several nucleoside DNA chain-terminating drugs were positively correlated with

XIAP protein levels, suggesting that drug sensitivity may be associated with expression of this protein (Table 1A). The most significant correlation was between XIAP protein levels and *in vitro* chemosensitivity to Cytarabine, indicating a possible role for XIAP in conferring sensitivity to this agent *in vitro* (Pearson Correlation Coefficient=0.44; p (two-tail) < 0.0006). For cIAP1 the most significant correlation was seen between cIAP1 protein levels and *in vitro* chemoresistance to Carboplatin ($p < 0.002$) and Cisplatin ($p < 0.002$) (Table 1B). Significant associations between higher levels of cIAP1 protein and resistance to several DNA alkylating agents and the topoisomerase inhibitor VP16 were also observed (Table 1B). A complete list of the tested cytostatic drugs is available through the internet at: http://epnws1.ncicrf.gov:2345/dis3d/dtp.html. No significant correlations with drug cytotoxicity data and IAP-family mRNA levels were found.

A comparison of the relative levels of IAP-family mRNAs and proteins with the expression of more than 30 genes previously evaluated in the NCI 60 tumor cell line screening panel failed to reveal any significant correlations (Pearson: - 0.10). This search for correlations included e.g. p53, Bcl-2, Jun, Fos, Ras and Rb. A complete list of the gene targets is available through the internet at: http://epnws1.ncicrf.gov:2345/dis3d/dtp.html.

Comparisons of IAP Expression with Clinical Response to Chemotherapy in AML. The observed correlation of higher levels of XIAP protein with greater sensitivity to nucleoside analog drugs was paradoxical in terms of a priori expections based on gene transfection evidence that over-expression of XIAP protects tumor cell lines from

apoptosis induced by various anticancer drugs (reviewed in 33, 36). Since XIAP protein levels showed the greatest correlation with cytarabine (AraC), we explored the relation of XIAP expression to *in vivo* drug responses in a clinical context where AraC-based therapy is standardly employed, namely in patients with AML (34). All patients were treated with high-dose Ara-C containing regimen. The relative levels of XIAP protein were compared with clinical responses to chemotherapy, consisting of high-dose cytarabine plus idarubicine, using leukemia cell samples derived from 78 AML patients. Expression of XIAP protein was detected by immunoblotting in 76 of 78 samples tested, however the level of XIAP was very low (<5% of the control cell line) in 20 cases. The range of expression of XIAP was heterogeneous across all FAB and cytogenetic categories, except for promyelocytic leukemia where there was a narrow range of expression (not shown). Bcl-2, Bax, Caspase-2 and Caspase-3 expression have been previously measured for most of these patients (7, 25, 27). No significant correlation between expression of these other apoptosis-related proteins and XIAP protein levels was found (data not shown).

To assess possible threshold effects of XIAP protein on survival, while avoiding searching for optimal cut-points for expression, results were rank-ordered and then divided into thirds. Because data derived from patients with XIAP levels in the top two-thirds were similar (not shown), they were collapsed into one arm for further analysis and comparison. Patients with lower XIAP levels had significantly longer survival (median survival 133 vs. 52.5 weeks, P = 0.05) and a tendency towards longer median remission duration (87 vs. 52.5 weeks, P = 0.13) than those with higher levels of XIAP. (Table 2 and Figure 4). For example, 42% of the patients with low XIAP expression are alive today,

as compared to 23% with high XIAP expression (P = 0.004) (Table 2). Patients with low levels of XIAP had only a slightly higher complete remission (CR) rate with induction chemotherapy (73% vs. 65%, P=0.42) but a lower relapse rate (RR) (42% vs. 65%, P = 0.17) compared to patients with XIAP levels in the top two-thirds (Table 2). However, neither the CR rate nor RR data reached statistical significance. No statistically significant differences were noted between the XIAP high and low groups with respect to age, cytogenetics, frequency of an antecedent hematological disorder or gender, however patients with higher XIAP were more likely to have poor Zubrod performance status (Table 2).

From this evaluation, we conclude that higher levels of XIAP protein tend to be associated with shorter remission durations and shorter overall survival in AML patients treated with cytarabine-containing regimens. Thus, these findings are at odds with *in vitro* correlations derived from analysis of the NCI 60 cell screening panel, and are fitting with expections based on knowledge of XIAP's anti-apoptotic activity in cells.

Discussion

In this study, we show that expression of several members of the human IAP family of anti-apoptotic genes is differentially regulated within the NCI-panel of 60 human tumor cell lines and in freshly isolated human leukemia samples. XIAP and c-IAP1 were widely expressed in tumor cell lines. However, the relative levels of these IAP family members were variable among the tumor lines evaluated, implying differential regulation of the expression of these anti-apoptotic genes within cancers. In contrast to XIAP and cIAP1, the expression of c-IAP2 appears to be more restricted since cIAP2 mRNA was detectable in only approximately half of the analyzed tumor cell lines, and cIAP2 protein was found at detectable levels in only 5 (8%) of tumor cell lines. In comparison, NAIP mRNA was undetectable in all tumor cell lines tested. Thus, this particular member of the IAP family appears not be to a major participant in solid tumors. The IAP-family member Survivin has been shown previously to be widely expressed in the tumor cell lines of the NCI 60 cell line screening panel (18).

Interestingly, IAP mRNA and protein levels were commonly non-congruent, suggesting the likelihood of post-transcriptional regulation of the expression of these anti-apoptotic genes, perhaps through translational mechanisms or by differential rates of protein turnover. In this regard, evidence of translational control of XIAP has recently been reported, revealing the presence of an internal ribosome entry site (IRES) within the XIAP mRNA (35). This lack of correlation between XIAP, cIAP1, and cIAP2 has important implications with respect to any future attempts to use cDNA arrays or related technologies for assessing IAP-family gene expression in cancers. The finding that cIAP1 protein but not cIAP1 mRNA levels were associated with resistance to

several anticancer drugs among the tumor cell lines of the NCI 60 cell screening panel lends additonal support to the argument that measurements of protein and not mRNA levels is critical to understanding the role of this family of anti-apoptotic genes in cancer.

The variability in IAP protein levels seen here among the NCI's 60 human tumor cell lines suggests that further correlative studies of IAP expression with clinical outcome and with other biomarkers should be informative with regards to assessing the prognostic significance of these anti-apoptotic proteins. Moreover, the lack of correlation of IAPs with other known tumor-related genes previously assessed in the NCI 60 cell panel (e.g., c-Jun, Ras, Fos, p53, Rb), raises the possibility that IAPs could serve as independent risk factors for some types of malignancies, as recently suggested for Survivin (9,15,21).

The paradoxical association of XIAP protein levels with sensitivity rather than resistance to cytarabine and some other nucleoside analogs among the NCI panel of 60 tumor cell lines remains unexplained. We might speculate that a fortuitous correlation exists between XIAP and levels of other proteins that might sensitize cells to such agents, such as nucleotide kinases and phosphatases. Moreover, the correlation between XIAP levels and sensitivity of cell lines to various drugs *in vitro* does not take into account the clinical usefulness of this approach: e.g. Ara-c is not the cytotoxic agent of choice for solid tumors. Given the overwhelming evidence that XIAP is a caspase-inhibiting, anti-apoptotic protein (reviewed in 33, 36), this observation illustrates the difficulty in drawing conclusions about protein function from correlative approaches. In this regard, the NCI 60 tumor cell line panel was assembled originally for the purpose of identifying compounds with potential antitumor activity (37). In recent years, however, attempts

have been made to use bioinformatics and genomics technologies to reveal associations between cytotoxic responses of cancer cell lines to compounds and various biomarkers. Future efforts of this type may ultimately provide a molecular explanation for the paradoxical correlation of XIAP protein with greater sensitivity to nucleoside agents.

Though little attempt has been made thus far to compare the levels of IAPs in tumors with other biomarkers or with clinical outcome, it has been reported that Survivin expression in neuroblastomas correlates with clinically more aggressive, histologically unfavorable disease (21). Moreover, higher levels of Survivin protein as determined by immunostaining and p53 accumulation (indicative of mutant p53) were positively correlated in a survey of gastric cancers (9), implying an association of Survivin with more aggressive disease.

Prompted by an unexpected inverse correlation between XIAP protein levels and resistance to cytarabine in the NCI 60 cell screening panel, we compared the expression of this IAP family members in AML blasts derived from newly diagnosed, untreated patients, making correlations with clinical outcome. Patients with low levels of XIAP (lowest third) enjoyed a significantly longer survival and tended to have longer median remission durations. These data suggest that XIAP may have prognostic potential in AML, a finding which should be evaluated in additional studies involving larger numbers of AML patients and extended to other types of cancer. Moreover, these *in vivo* data are fitting with expections based on knowledge of XIAP's anti-apoptotic activity in cells.

These results thus warrant further research on both the functions and prognostic relevance of XIAP and other IAP-family genes in cancer and leukemia.

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Figure Legends

Figure 1. IAP-Family mRNA Levels in the NCI Panel of Tumor Cell Lines: Examples of RNase Protection Assays. ^{32}P-labeled anti-sense RNA probes for XIAP, NAIP, c-IAP1, c-IAP2 and GAPDH were hybridized with target human mRNAs (12 μg) from the NCI panel of tumor cell lines. The remaining "RNase-protected" probes were purified, resolved on denaturing polyacylamide gels according to their size, and imaged by autoradiography. Examples are shown for several human tumor cell lines.

Figure 2. IAP-Family mRNA Levels in the NCI Panel of Tumor Cell Lines: Graphical Summary of Results. Graphical summary of the XIAP (Figure 2A), c-IAP1 (Figure 2B), and c-IAP2 (Figure 2C) mRNA expression levels in the form of a mean-graph, where expression above average is drawn to the right of the center line and levels of specific mRNAs below the mean are drawn to the left of the center line. The cell lines are grouped according to type. "Deltaval sum" is the expression value of IAPs (relative expression) for each cell line after subtracting the mean of that IAP's expression as determined from all the cell lines analyzed. A "Deltaval sum" of -1.8S for cIAP2 equals not expressed.

Figure 3. IAP-Family Protein Levels in the NCI Panel of Tumor Cell Lines. (A) Examples of immunoblot data are shown for several human tumor cell lines using monoclonal antibodies specific for XIAP, cIAP1 and cIAP2. In all cases, lysates were normalized for total protein

content (50 μg/lane). (B) Graphical summary is presented XIAP and cIAP1 protein levels in the form of a mean-graph, where expression above average is drawn to the right of the center line and XIAP protein levels below the mean are graphed to the left of the center line. The cell lines are grouped depending tumor-type. "Deltaval sum" is the expression value of XIAP (relative expression) for a given cell line after subtracting the mean of XIAP expression as determined from analysis of all 60 cell lines.

Figure 4. Effect of XIAP Protein Expression on AML Patient Survival and Remission Duration. The plots show Kaplan-Meier survival (A) and remission duration (B) for 79 AML patients stratified according to XIAP protein levels. The cohort was divided into thirds based on rank-order analysis of XIAP protein levels. As discussed in the text, the top two-thirds were collapsed into one group.

Table 1. Correlation of IAP Protein Levels with Chemosensitivity Data from the NCI 60 Cell Screening Panel. Comparison of IAP protein levels and chemosensitivity data for the 60 cell line panel (NCI) resulted in a rank-order correlation. Representative results are shown for XIAP (Table 1A) and cIAP1 (Table 1B) protein levels. Positive correlation indicates that a greater abundance of the target may be associated with sensitivity to the drug, while a negative correlation is indicative of more target conferring cellular resistance to the given drug. Pearson correlation coefficient and two tail p-values were used to assign possible significance to these data.

Table 2: Clinical Characteristics of AML Patients. The cohort was divided into thirds based on rank-order analysis of XIAP protein levels. As discussed in the text, the top two-thirds were collapsed into one group. Abbreviation: N = number of patients, 1 = patients with blasts>15%, 2 = compares lower third to top two thirds

Table 1A: XIAP Protein Correlation with Cytotoxic Drugs

Rank	Name of Drug	Pearson	P (two-tail)
1	Cytarabine	0.44	0.00059
2	Hydrazine Sulfate	0.41	0.00123
3	Emofolin Sodium	0.39	0.00211
4	Cyclocytidine	0.38	0.00296
5	3-Deazaguanine	0.34	0.00835
6	Flavone Acetic Acid	0.32	0.01098
7	Deoxyspergualin	0.32	0.01136
8	Aclacinomycin A	0.31	0.01604
9	Pyrazoloacridine	0.31	0.01672
10	Rubidazone	0.30	0.01794
.			
21	Fludarabine-PO_4	0.26	0.04168
.			
27	VP-16	0.24	0.06258
.			
32	Methotrexate	0.23	0.06958
.			
62	Flavoperidol	0.19	0.14559
.			
166	Tamoxifen	0.02	0.82223

Table 1B: cIAP1 Protein Correlation with Cytotoxic Drugs

Rank	Name of Drug	Pearson	P (two-tail)
1	Carboplatin	-0.41	0.00114
2	Cis-Platinium	-0.39	0.00195
3	Asaley	-0.39	0.00209
.			
8	Methyl CCNU	-0.34	0.00740
9	VP-16	-0.34	0.00769
10	Thioguanine	-0.33	0.00946
.			
15	Bleomycin	-0.31	0.01471
.			
17	Cytarabine	-0.31	0.01628
.			
19	Chlorambucil	-0.30	0.01844

.

Table 1A: XIAP Protein Correlation with Cytotoxic Drugs

Rank	Name of Drug	Pearson	P (two-tail)
1	Cytarabine	0.44	0.00059
2	Hydrazine Sulfate	0.41	0.00123
3	Emofolin Sodium	0.39	0.00211
4	Cyclocytidine	0.38	0.00296
5	3-Deazaguanine	0.34	0.00835
6	Flavone Acetic Acid	0.32	0.01098
7	Deoxyspergualin	0.32	0.01136
8	Aclacinomycin A	0.31	0.01604
9	Pyrazoloacridine	0.31	0.01672
10	Rubidazone	0.30	0.01794
.			
21	Fludarabine-PO_4	0.26	0.04168
.			
27	VP-16	0.24	0.06258
.			
32	Methotrexate	0.23	0.06958
.			
62	Flavoperidol	0.19	0.14559
.			
166	Tamoxifen	0.02	0.82223

Table 1B: cIAP1 Protein Correlation with Cytotoxic Drugs

Rank	Name of Drug	Pearson	P (two-tail)
1	Carboplatin	-0.41	0.00114
2	Cis-Platinium	-0.39	0.00195
3	Asaley	-0.39	0.00209
.			
8	Methyl CCNU	-0.34	0.00740
9	VP-16	-0.34	0.00769
10	Thioguanine	-0.33	0.00946
.			
15	Bleomycin	-0.31	0.01471
.			
17	Cytarabine	-0.31	0.01628
.			
19	Chlorambucil	-0.30	0.01844

.

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Table 2

Variable	All Patients[1]	XIAP Low	XIAP High	P-value[2] (%)
N	78	26	52	
Male : Female	44 : 34	12 : 14	32 : 20	.20
Age (Median)	52	49	54	.10
Cytogenetics: Favorable Intermediate Unfavorable	12 35 36	03 15 08	09 20 28	.27
Antecedent Hematological Disorder > 2 months (%)	18	12	21	.29
Zubrod Performance Status 3 or 4 (%)	12	00	17	.02
Complete Remission (%)	68	73	65	.42
Relapse (%)	55	42	65	.17
Alive (%)	29	42	23	.004
Median Survival (Weeks)	67	133	52	.05
Median Remission Duration (Weeks)	57	87	53	.13

FIGURE 1

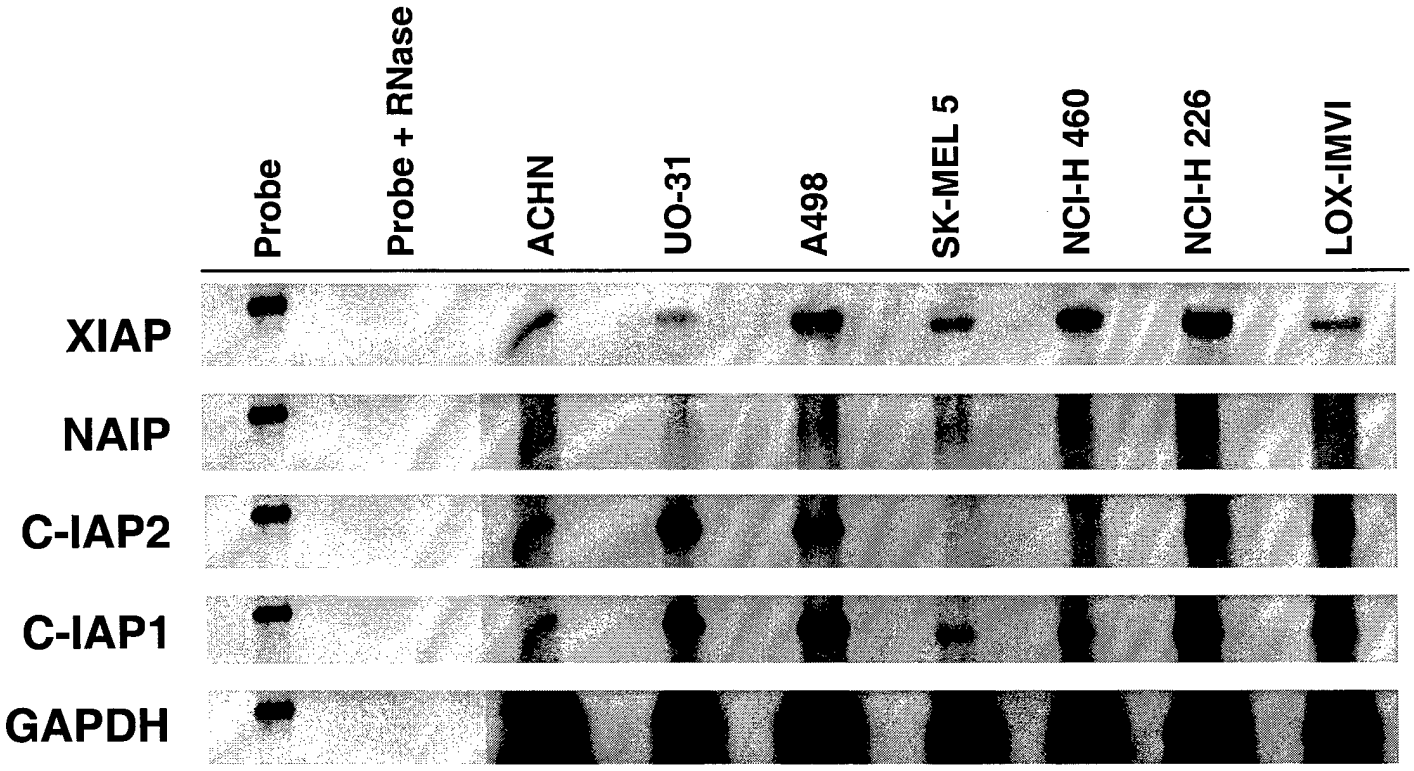

Probe
Probe + RNase
ACHN
UO-31
A498
SK-MEL 5
NCI-H 460
NCI-H 226
LOX-IMVI
XIAP
NAIP
C-IAP2
C-IAP1
GAPDH

FIGURE 2

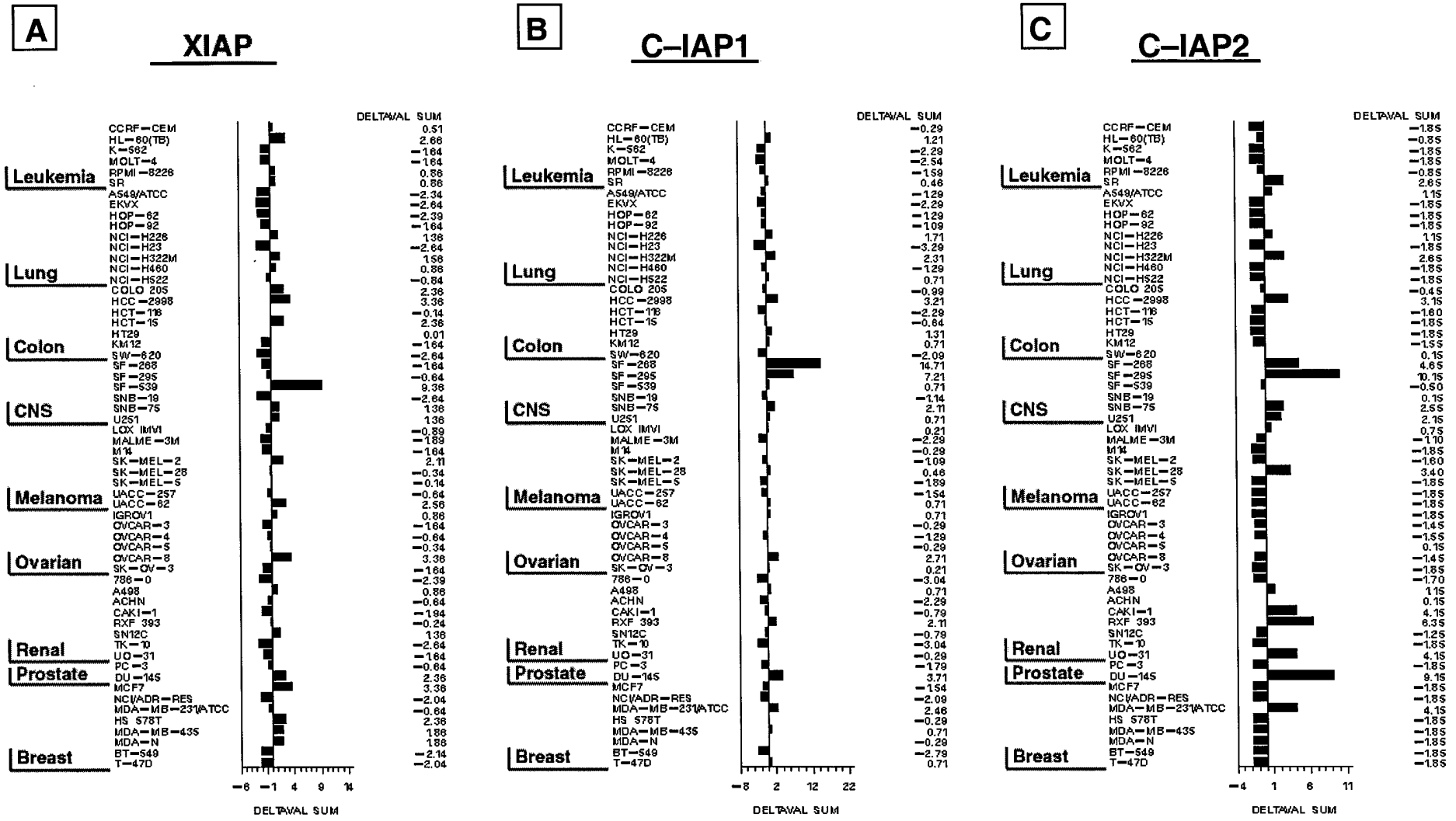
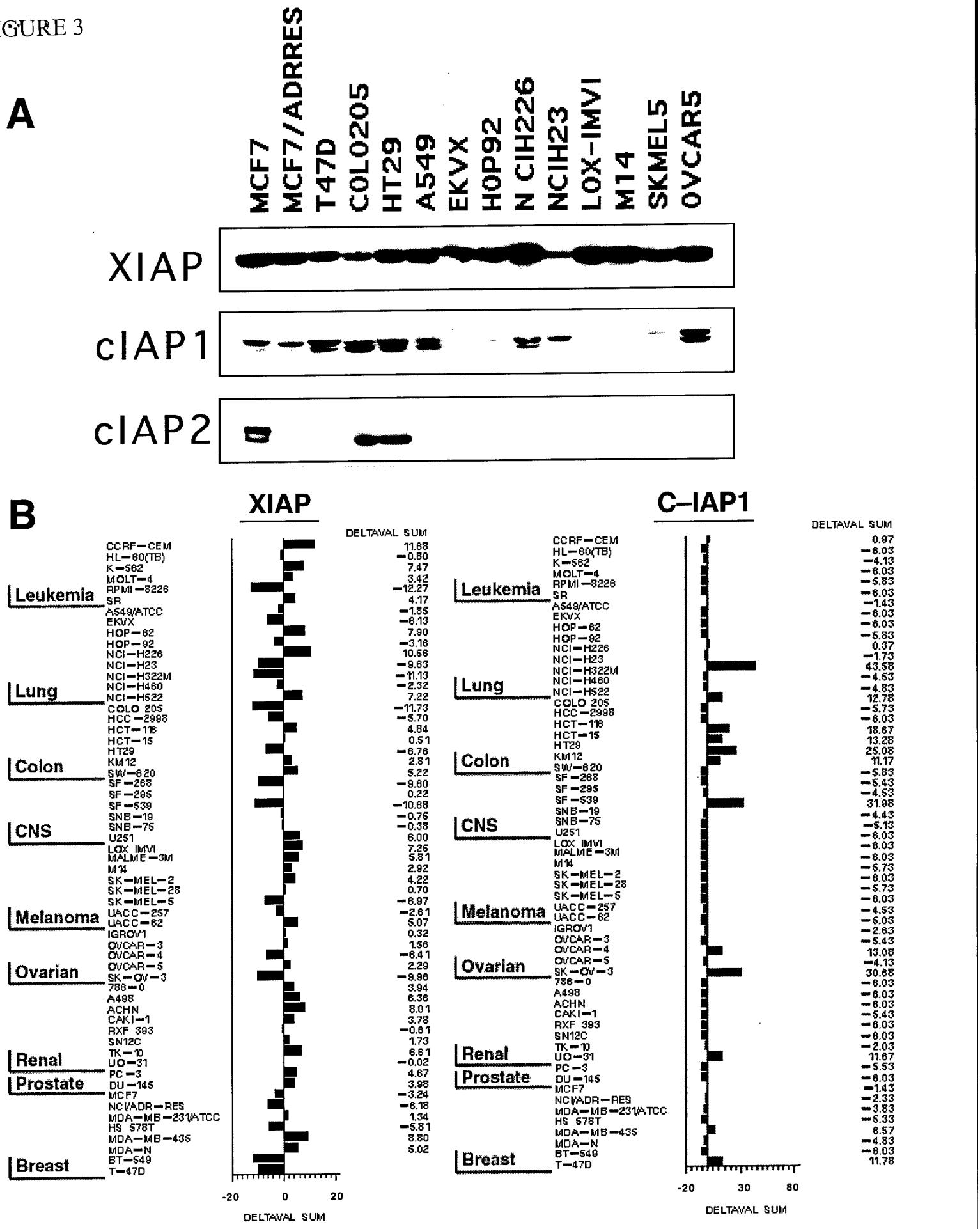

A
XIAP
B
C-IAP1
C
C-IAP2
DELTAVAL SUM
Leukemia
Lung
Colon
CNS
Melanoma
Ovarian
Renal
Prostate
Breast
CCRF-CEM
HL-60(TB)
K-562
MOLT-4
RPMI-8226
SR
A549/ATCC
EKVX
HOP-62
HOP-92
NCI-H226
NCI-H23
NCI-H322M
NCI-H460
NCI-H522
COLO 205
HCC-2998
HCT-116
HCT-15
HT29
KM12
SW-620
SF-268
SF-295
SF-539
SNB-19
SNB-75
U251
LOX IMVI
MALME-3M
M14
SK-MEL-2
SK-MEL-28
SK-MEL-5
UACC-257
UACC-62
IGROV1
OVCAR-3
OVCAR-4
OVCAR-5
OVCAR-8
SK-OV-3
786-0
A498
ACHN
CAKI-1
RXF 393
SN12C
TK-10
UO-31
PC-3
DU-145
MCF7
NCI/ADR-RES
MDA-MB-231/ATCC
HS 578T
MDA-MB-435
MDA-N
BT-549
T-47D
0.51 2.66 -1.64 -1.64 0.86 0.86 -2.34 -2.64 -2.39 -1.64 1.36 -2.64 1.58 0.86 -0.84 2.36 3.36 -0.14 2.36 0.01 -1.64 -2.64 -1.64 -0.64 9.36 -2.64 1.36 1.36 -0.89 -1.89 -1.64 2.11 -0.34 -0.14 -0.64 2.56 0.86 -1.64 -0.64 -0.34 3.36 -1.64 -2.39 0.86 -0.64 -1.94 -0.24 1.36 -2.64 -1.64 -0.64 2.36 3.36 -2.04 -0.64 2.36 1.86 1.88 -2.14 -2.04
-6 -1 4 9 14
DELTAVAL SUM
-0.29 1.21 -2.29 -2.54 -1.59 0.46 -1.29 -2.29 -1.29 -1.09 1.71 -3.29 2.31 -1.29 0.71 -0.99 3.21 -2.29 -0.84 1.31 0.71 -2.09 14.71 7.21 0.71 -1.14 2.11 0.71 0.21 -2.29 -0.29 -1.09 0.46 -1.89 -1.54 0.71 0.71 -0.29 -1.29 -0.29 2.71 0.21 -3.04 0.71 -2.29 -0.79 2.11 -0.79 -3.04 -0.29 -1.79 3.71 -1.54 -2.09 2.46 -0.29 0.71 -0.29 -2.79 0.71
-8 2 12 22
DELTAVAL SUM
-1.8S -0.8S -1.8S -1.8S -0.8S 2.6S 1.1S -1.8S -1.8S -1.8S 1.1S -1.8S 2.6S -1.8S -1.8S -0.4S 3.1S -1.60 -1.8S -1.5S 0.1S 4.6S 10.1S -0.50 0.1S 2.5S 2.1S 0.7S -1.10 -1.8S -1.60 3.40 -1.8S -1.8S -1.8S -1.4S -1.5S 0.1S -1.4S -1.8S -1.70 1.1S 0.1S 4.1S 6.3S -1.2S -1.8S 4.1S -1.8S 9.1S -1.8S 4.1S -1.8S -1.8S -1.8S -1.8S
-4 1 6 11
DELTAVAL SUM

FIGURE 3


A
MCF7
MCF7/ADRRES
T47D
COL0205
HT29
A549
EKVX
H0P92
N CIH226
NCIH23
L0X-IMVI
M14
SKMEL5
OVCAR5
XIAP
cIAP1
cIAP2
B
XIAP
C-IAP1
DELTAVAL SUM
Leukemia
Lung
Colon
CNS
Melanoma
Ovarian
Renal
Prostate
Breast
-20
0
20
30
80

A

FIGURE 4

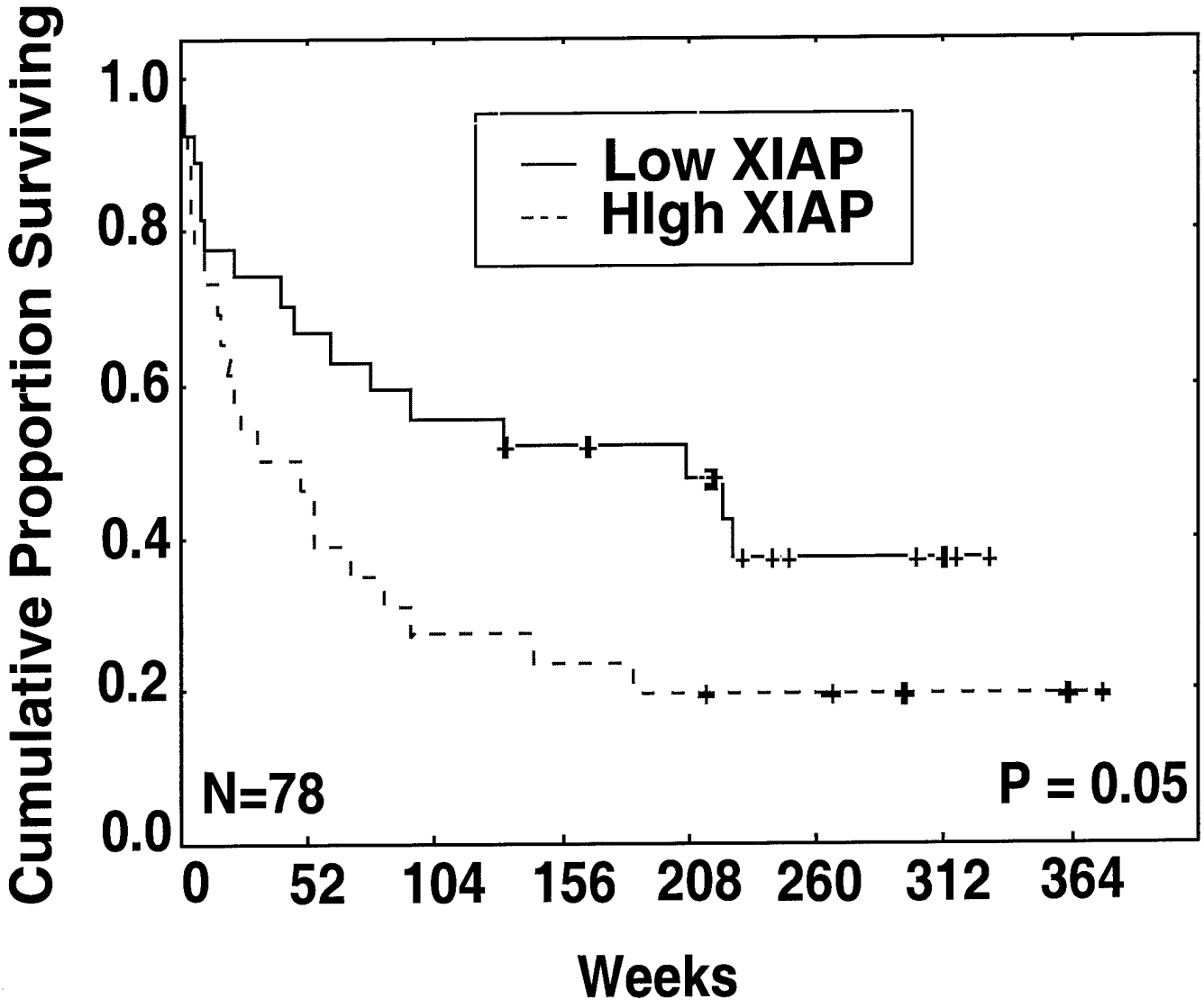
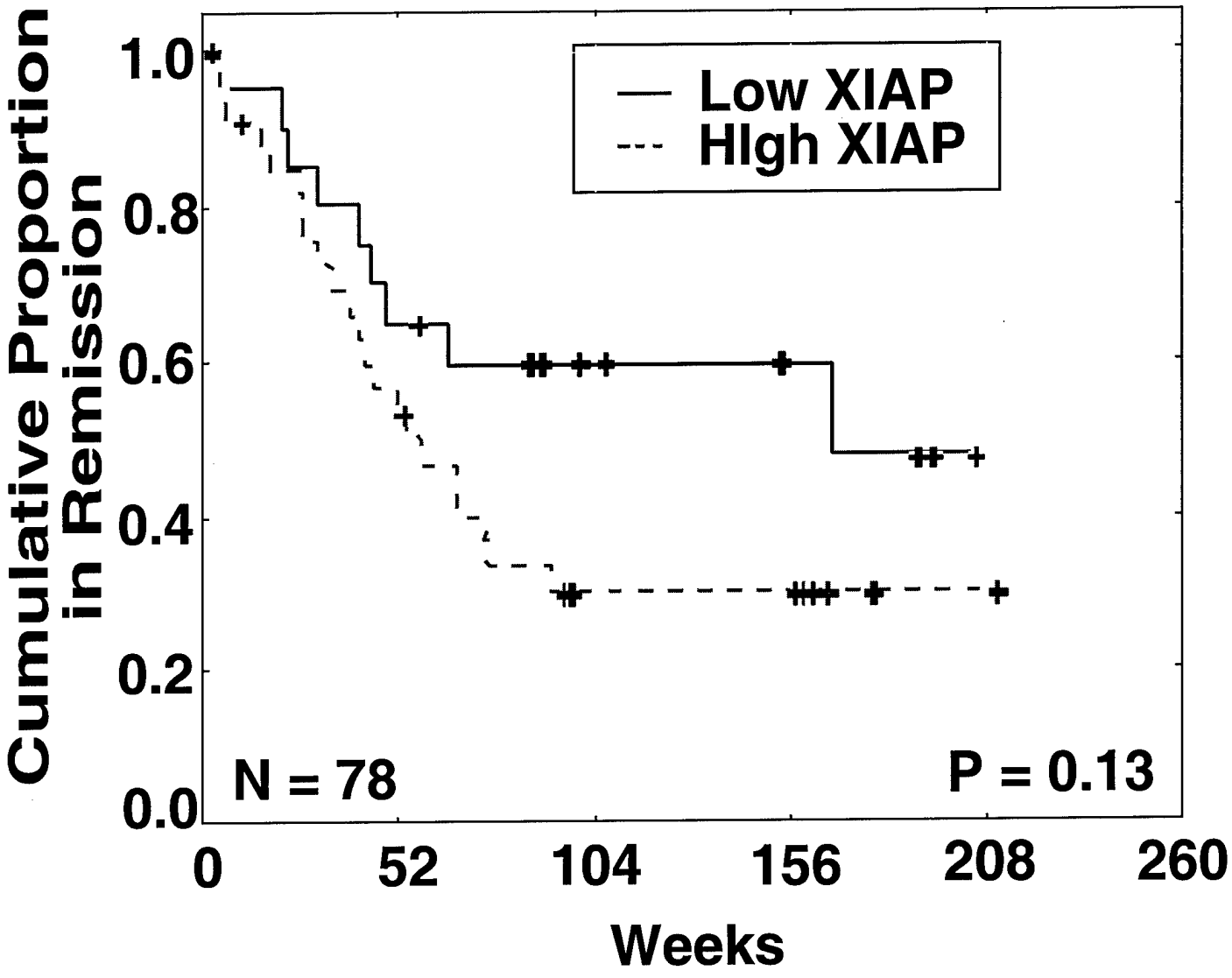

Cumulative Proportion Surviving
1.0
0.8
0.6
0.4
0.2
0.0
Low XIAP
HIgh XIAP
N=78
P = 0.05
0
52
104
156
208
260
312
364
Weeks

FIGURE 4B


Cumulative Proportion in Remission
1.0
0.8
0.6
0.4
0.2
0.0
Low XIAP
HIgh XIAP
N = 78
P = 0.13
0
52
104
156
208
260
Weeks